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Phosphatome-wide siRNA screen in 3T3 cells

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We use this protocol and it's working

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Abstract

This protocol describes how we performed a phosphatome-wide siRNA screen in 3T3 cells to look for LRRK2-counteracting phosphatases for pRab12 and pRab10.

Attachments

XLSX

phosphatase screen g...

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Materials

Dharmacon mouse phosphatase library, OnTarget SmartPool (G113705 and custom library, see attached sheet for full list of genes)
siRNA resuspension buffer (Dharmacon B-002000-UB-100)
OnTarget SmartPool non-targeting siRNA (Dharmacon D-001810-10)
OnTarget SmartPool mouse Rab12 siRNA (Dharmacon L-040865-01)
3T3 Flp-In cells (Invitrogen R76107)
Opti-MEM (Gibco 31985088)
Dharmafect 1 (Dharmacon T-2001)
DMEM (Cytiva SH30243.02)
FBS (Sigma F0926)
Penicillin-Streptomycin (Sigma P4333)
6-well plates (FB 012927)
2 mL 96 well assay plates (Costar 3960)
rabbit anti-pRab10 (ab230261)
rabbit anti-pRab12 (ab256487)
mouse anti-total Rab10 (ab104859)
mouse anti-total Rab12 (sc-515613)
4–20% Criterion TGX Precast Midi Protein Gel (Bio-Rad 56710954)

Preparation of siRNA library

1 Obtain mouse phosphatome-wide siRNA library (Dharmacon, OnTarget SmartPool, 0.5 nmol), provided in a 96 well format.

2 Resuspend siRNA library in 25 µL 1X siRNA buffer (Dharmacon) per well, using a multichannel pipette, to achieve a final concentration of [M] 20 micromolar (µM) .

3 Prepare siRNA controls (non-targeting, Rab12) by resuspension using 1x siRNA buffer to a concentration of [M] 20 micromolar (µM) .

siRNA transfection

4 Seed 2×10^5 3T3 Flp In cells/well in each well of a 6-well plate using 1.6 mL complete DMEM (DMEM+10% FBS+1% Pen-Strep). Plate cells into a sufficient number of wells to accommodate the total number of siRNAs plus control wells (~336 wells or 56, 6-well plates). Cell counts will need to be optimized for each cell line to determine optimal confluency at time of transfection.

5 16-24 hours later, ensure cells are at ~30% appropriate confluency for transfection.

6 In a deep well (2 mL) 96 well assay plate, add 198 µL Opti-MEM to each well. Then add 2.5 µL of [M] 20 micromolar (µM) siRNA. This will achieve a final concentration of 25 nM during the eventual transfection). Incubate 00:05:00 at RT.

7 Make a master mix for the control siRNAs, scaling up the amounts from step 6 above. For example, for 8 transfection reactions, use 20 µL siRNA in 1.580 mL Opti-MEM. Make sufficient non-targeting control samples to enable treatment with MLi-2 (LRRK2 inhibitor) at a later stage.

8 Make a second master mix of Opti-MEM and Dharmafect 1 (Dharmacon) transfection reagent, sufficient for all transfections. We use 4 µL Dharmafect per transfection, in total volume of 200 µL Opti-MEM per transfection. For 350 transfections, use 1.4 mL

Dharmafect + 68.6 mL Optimem for a total of 70 mL . Mix and incubate 00:05:00 at RT.

9 Using a multichannel pipette, add 200 µL of OptiMEM-Dharmafect mixture to each well containing diluted siRNAs (from step 6). Mix and incubate for 00:20:00 at RT.

10 Aliquot 400 µL of each siRNA+Dharmafect+Opti-MEM mixture into a well of a 6-well plate containing seeded cells.

11 24 hours after transfection, replace media with fresh, complete DMEM.

Analysis

12 72 hours after transfection, cells are ready to be harvested for immunoblotting or other analysis.
Treat with MLi-2 in some of the wells containing non-targeting siRNA as a control. Add 2 µL of [M] 100 micromolar (µM) MLi-2 to the 2 mL of media in each well of 6-well plate (1:1000 dilution) to achieve final concentration of 100 nM MLi-2. Incubate for 02:00:00 at 37°C

13 For each well in 6-well plate containing siRNA, add [M] 100 nanomolar (nM) MLi-2 for 00:20:00 just prior to harvesting. Do this in batches to ensure accurate kinetics; add MLi-2 to a few 6-well plates and then collect those. If you are not able to collect immediately, put plates on metal plate on ice and store at 4°C.

14 See protocol (**dx.doi.org/10.17504/protocols.io.bsgrnbv6**) for harvesting, lysing, and immunoblotting of cell lysates to analyze pRab10, total Rab10 and pRab12, total Rab12 levels. Protein lysates are resolved on a 4–20% Criterion TGX Precast Midi Protein Gel (Bio-Rad) and transferred onto nitrocellulose membranes as described.

For antibodies, pRab10 (ab230261) and pRab12 (ab256487) antibodies can be multiplexed; total Rab10 (ab104859) and total Rab12 (sc-515613) antibodies can also be multiplexed since antibodies are specific enough and Rab10 and Rab12 sizes can be differentiated on a blot. All primary antibodies are detected using LICOR secondary antibodies and visualized using the LICOR Odyssey DLx imager. Protein levels are analyzed using the gel scanning plugin on ImageJ.